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Indian Standard

METHODS OF TEST FOR DAIRY INDUSTRY

PART III BACTERIOLOGICAL ANALYSIS OF MILK

(*First Revision*)

First Reprint AUGUST 1990

UDC 637·127·6 : 576·8·078

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BUREAU OF INDIAN STANDARDS
MANAK BHAVAN, 9 BAHADUR SHAH ZAFAR MARG
NEW DELHI 110002

Gr 7

July 1978

Indian Standard

METHODS OF TEST FOR DAIRY INDUSTRY

PART III BACTERIOLOGICAL ANALYSIS OF MILK

(First Revision)

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(*Continued on page* 2)

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Indian Standard

METHODS OF TEST FOR DAIRY INDUSTRY

PART III BACTERIOLOGICAL ANALYSIS OF MILK

(First Revision)

0. FOREWORD

0.1 This Indian Standard (Part III) (First Revision) was adopted by the Indian Standards Institution on 28 December 1977, after the draft finalized by the Dairy Products Sectional Committee had been approved by the Agricultural and Food Products Division Council.

0.2 This standard (Part III) covers methods commonly used for detailed bacteriological analysis of milk. The other parts are:

Part I Rapid examination of milk
Part II Chemical analysis of milk
Part IV Freezing point depression of milk
Part V Methods of dairy plant control

0.3 This standard was first published in 1962 and is being revised in order to make the methods of bacteriological analysis of milk more precise and up-to-date. It is expected that adoption of this standard would help in achieving uniformity in the methods of bacteriological analysis of milk, thereby facilitating the interpretation and comparison of results.

0.4 For preparation of media for microbiological work, it is recommended that ingredients of proper quality conforming to relevant Indian Standards are used. A list of the Indian Standards is given in Appendix A.

0.5 In reporting the result of a test or analysis made in accordance with this standard, if the final value, observed or calculated is to be rounded off, it shall be done in accordance with IS : 2-1960*.

1. SCOPE

1.1 This standard (Part III) prescribes methods commonly used for detailed bacteriological analysis of milk. It also includes the methods of bacteriological analysis for special purposes.

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*Rules for rounding off numerical values (*revised*).

1.1.1 The specific methods to be employed would depend upon the object of analysis.

2. COLLECTION OF MILK SAMPLES

2.1 Procedure for collection of samples shall be as prescribed in **3** of IS : 1479 (Part I)-1960*.

3. METHYLENE BLUE REDUCTION TEST (MBRT)

3.1 Apparatus, Reagents and Procedure — Details of the apparatus, reagents and general procedure shall be the same as described in **12** of IS : 1479 (Part I)-1960*. The tubes shall, however, be incubated beyond half an hour until the milk is completely decolourized or decolourized up to 5 mm from the surface. The tubes shall be observed and inverted initially at half hour and at hourly intervals thereafter. If the milk begins to decolourize, the tubes shall not be inverted. Record the time at which complete decolourization is observed.

3.2 Recording of Results — During incubation, observe colour changes as follows:

a) If any sample is decolourized on incubation for 30 minutes, record the reduction time as MBRT-30 minutes.

b) Record such readings as reduction times in whole hours. For example, if the colour disappears between 0·5 and 1·5 hour readings, record the result as MBRT-1 hour; similarly, if between 1·5 and 2·5 hours as MBRT-2 hour and so on.

c) Immediately after each reading, remove and record all the decolourized samples and then gently invert the remaining tubes if the decolourization has not yet begun.

NOTE 1 — Optionally use an interval timer to remind the observer when successive examinations should be made.

NOTE 2 — Because of uneven disappearance of blue colour, that may be noted at the final reading time, record the sample as decolourized when only a faint blue ring (about 5 mm) persists at the top.

3.3 Grading — The following methylene blue reduction times are suggested as guide for grading of raw milk supplies:

Methylene Blue Reduction Time in Hours	*Grade*
5 and above	Very good
3 and 4	Good
1 and 2	Fair
½	Poor

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*Methods of test for dairy industry: Part I Rapid examination of milk.

4. ONE-HOUR RESAZURIN TEST

4.1 This test gives a better indication of the quality of milk than the 10-minute resazurin test described in **11** of IS : 1479 (Part I)-1960*.

4.2 Apparatus, Reagents and Procedure — Details of the apparatus, reagents and procedure shall be the same as described in **11** of IS : 1479 (Part I)-1960*, except that the tubes are examined after incubation at 37·0 ± 0·5°C for one hour.

4.3 Interpretation — The following standards are suggested as a guide for grading of raw milk supplies:

Resazurin Disc Number	*Grade*
4 or higher	Good
3·5 to 1	Fair
0·5 and 0	Poor

5. PLATE COUNT (PC)

5.1 This method is used for determining the total number of viable bacteria in milk and consists of mixing appropriate quantity of milk with a suitable nutrient agar medium in a petri dish and counting the bacterial colonies developed after incubation at a specified temperature for a definite period of time.

NOTE 1 — Although this method is time consuming and comparatively more expensive, it has been widely used with satisfactory results and is particularly suitable where low bacterial population is expected.

NOTE 2 — This method is especially useful for pasteurized milk and for line testing at various stages of processing for detecting sources of contamination. This test should be done in a well-ventilated room, free from dust and where microbial contamination of air (bacteria, yeasts and moulds) as determined by exposure of poured plates, shall not exceed 20 colonies per plate after a 15-minute exposure.

5.2 The plate count of bacteria in milk shall be determined according to IS : 5402-1969†. However, the samples for plating shall be prepared as given in **5.3** and test portions shall be measured as given in **5.4**.

5.3 Preparing Samples for Plating — Immediately before removal of any portion of any sample, vigorously mix the contents of each container until thorough mixing is assured. Invert the filled retail containers repeatedly until contents are homogeneous before removal of test portions. Before opening a sample container, remove from closure all material which may contaminate the sample. Where necessary, wipe the top of unopened container, with sterile cloth saturated with alcohol.

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*Methods of test for dairy industry: Part I Rapid examination of milk.
†Method for plate count of bacteria in foodstuffs.

5.3.1 Immediately before transferring each test portion of milk (except from filled containers as noted above) and of dilutions thereof, shake each container through 25 complete up and down movements of about 400 mm in 7 seconds.

5.4 Measuring Test Portions — When measuring milk, complete each transfer within 2 to 3 seconds by letting the column drain from the graduation mark to the apparent rest point of liquid in the tip of the pipette. Promptly and gently blow out the last drop. Make transfers carefully and do not rinse pipettes in dilution water. For maximum accuracy when preparing the 1 : 10 plate, dilute 11 ml of milk in a 99-ml blank, shake and transfer 1 ml thereof to the plate(s).

5.4.1 When measuring diluted milk, hold the pipette at an angle of about 45° with the tip touching the inside bottom of the petri dish or inside neck of the dilution bottle. Allow diluted milk to drain from the graduation mark to the apparent rest point in the tip of the pipette within 2 to 3 seconds and then touch once against a dry spot on glass. Do not blow out the drop in the tip of pipette.

After depositing test portions in each series of plates, pour the medium promptly.

5.5 Interpretation — The following criteria are suggested as a guide for grading of milk:

a) *Pasteurized Milk* (*at the plant in the final container*) — The plate count per millilitre (or gram) should not exceed 30 000.

b) *Raw Milk*

PC/ml (or g)	*Grade*
Not exceeding 200 000	Very good
Between 200 000 and 1 000 000	Good
Between 1 000 000 and 5 000 000	Fair
Over 5 000 000	Poor

6. LABORATORY PASTEURIZATION COUNT (LPC)

6.1 Presence of thermoduric (surviving but not growing at pasteurizing temperatures) and thermophilic (growing at and above pasteurizing temperatures) bacteria in milk indicates unhygienic conditions generally associated with careless practices and the use of unclean utensils and equipment. Thermoduric bacteria, while including some spore forming bacteria, are largely non spore-forming, heat-resistant types, growing on surfaces on ineffectively washed or improperly sanitized utensils on farms, in pre-heating equipment operated at favourable growth temperatures in processing plants and on inadequately sanitized pasteurizing equipment.

Control of thermoduric species is an operational problem involving both dairy farms and milk plants.

6.1.1 Thermophilic species are spore-forming, heat-loving types (growing best at about 55°C), and their presence in milk in large numbers indicates unduly long exposure to pasteurization temperature or in some instances, to other insanitary practices. Among practices which may result in this type of contamination are:

a) repasteurization of milk,
b) prolonged holding of milk or cream in vats at pasteurization temperatures,
c) stagnant milk in blind ends of piping at pasteurization temperatures,
d) continuous use of preheaters, long-flow holders or vats for more than 2 hours without periodically flushing out equipment with hot water,
e) passage of hot milk through filter cloths for more than 2 hours without replacing cloth,
f) residual foam on milk which remains in vats when emptied at the end of each 30-minute holding period, and
g) growth of thermophiles in milk residues on the surfaces of pasteurizing equipment.

6.1.1.1 Because of close relationship between the two groups of bacteria, methods for their detection may be identical except for the incubation of plates and tubes at 55°C, at which temperature only thermophilic types grow actively (*see* **6.7** for determination).

6.2 Application of Laboratory Pasteurization Count

6.2.1 The laboratory pasteurization count is a useful guide for detection of high thermoduric count of individual farm supplies. Even when milk from a few supplies containing excessive numbers of thermoduric species is diluted with milk from numerous supplies relatively free from these organisms, bacteria in the former may contribute appreciably toward high plate counts on retail samples of pasteurized milk. In such circumstances, laboratory pasteurization tests may be applied to samples of raw milk from individual producers to identify supplies containing heat-resistant bacteria. The method reveals unsatisfactory supplies not always shown by other tests, and is comparatively a simple procedure.

6.2.2 Before taking remedial measures, results of the laboratory pasteurization test should be fortified with a plate count of bacteria on a portion of the raw sample taken immediately before the heat treatment.

6.3 Collection of Samples

6.3.1 For detection of bacteria that survive pasteurization, normally use samples of producer's milk collected as described in **3** of IS : 1479 (Part I)-1960*.

6.3.2 For detection of thermophilic species, normally use samples of mixed milk before and after commercial pasteurization.

6.4 Apparatus and Materials

6.4.1 The apparatus and materials as described in **4** and **5** of IS : 5402-1969† are required. In addition, the following are also required:

a) *Vials or pasteurizing tubes* — screw cap vials, or tubes fitted with tight closures, size about 15 ml.

b) *Vials rack support* — corrosion-resistant, suggested size to accommodate 60 vials.

c) *Water-bath* — corrosion-resistant, suggested size about 25 × 25 × 30 cm suitably insulated and thermostatically controlled to maintain the desired temperature (expected 63·0 ± 0·2°C) with adequate stirring arrangement.

6.5 Laboratory Pasteurization Treatment — Shake the samples and transfer 5 ml to pasteurization tubes or vials, each individually identified with the sample source by number or by coded position in rack. Fill the tubes and vials so that the heat space between milk and closure is not less than 20 mm. Place the identified tubes in pasteurization rack(s). Immerse the tubes so that the water line is within 10 mm from top of tubes (about 10 mm above the milk line) and bring milk to 63·0 ± 0·2°C within 5 minutes after placing the rack in the bath. Use a pilot tube with accurate thermometer inserted therein for temperature determinations. Expose samples to pasteurizing temperature at not less than 63·0 ± 0·2°C for not less than 30 minutes. Avoid both over-heating and under-heating of test portions. Immediately cool the tubes in ice-water bath to 10°C. Without removal from the bath, shake the tubes vigorously. Prior to transfer of test portions, shake each sample.

6.6 Plating, Incubating and Counting Colonies for Thermoduric Bacteria

6.6.1 Plate test portions of samples, using appropriate dilutions (1 : 10, 1 : 100, 1 : 1 000, etc), incubate plates and count colonies as described in **17** of IS : 5402-1969†. Since counts so obtained depend upon exposure to specially controlled heat treatments of samples as against commercial pasteurization procedures, results should not be treated as plate counts. Results may be expressed as 'Laboratory Pasteurization Count per millilitre at the recording pasteurization temperature (LPC/ml............, at............°C)'.

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*Methods of test for dairy industry: Part I Rapid examination of milk.

†Method for plate count of bacteria in foodstuffs.

6.6.2 A laboratory pasteurization count may be compared with a plate count, made simultaneously on an unheated portion from the same sample.

6.6.3 *Interpretation* — Presence in individual producer's samples of appreciable numbers of bacteria that survive laboratory pasteurization indicates contamination from poorly sanitized farm utensils. Poorly sanitated processing equipment in milk plants may also be a source of contamination with thermoduric bacteria.

6.7 Determination of Thermophilic Count

6.7.1 Samples of mixed milk shall be collected before and after pasteurization in accordance with the procedure described in **3** of IS : 1479 (Part I)-1960*. The method of plating the samples and counting the colonies is the same as that described for thermoduric bacteria (*see* **6.6**) except that the poured plates are incubated at 55°C for 2 days. In order to prevent rapid drying of the agar medium in the plates at such high temperature, it is advisable to pour larger quantities of the medium into the plates than that used in the procedure for plate count and also to control humidity in the incubator by keeping a beaker of water.

6.7.2 *Interpretation* — Presence of a large number of thermophilic bacteria in pasteurized milk indicates heavy build-up of thermophilic bacteria in the pasteurization plant due to continuous running of milk at high temperature and improper cleaning and sanitation of the plant. High thermophilic counts in raw milk signify unhygienic conditions of production and handling of milk in inefficiently cleaned and sanitized utensils.

7. PSYCHROPHILIC BACTERIA

7.1 With increasing use of cold storage facilities for preservation of milk, the importance of detecting psychrophiles (cold-loving bacteria) assumes importance.

7.2 Contamination with psychrophilic bacteria in properly pasteurized milk may originate in water supplies used for rinsing equipment, or with residues or poorly sanitized equipment and containers, and subsequent growth of such contamination. Better sanitation of equipment and a reduction of the storage period before use after pasteurization are essentials of a good control programme. Psychrophilic types in dairy products may be detected and the relative numbers determined by incubating plates at 5°C for 7 days after plating samples as in **6.6**.

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*Methods of test for dairy industry: Part I Rapid examination of milk.

7.3 Interpretation — Presence of appreciable numbers of psychrophilic bacteria in properly pasteurized dairy products is an evidence of direct post-pasteurization contamination usually followed by growth of these species.

8. COLIFORM BACTERIA

8.1 General — This group of bacteria is important in quality control of milk as it is indicative of possible faecal contamination and due to its ability to produce acid and taints in milk. The presence of this group in milk considered to be an indicator of the degree of unhygienic practices during production, processing or storage and is intended to measure general care taken in handling this product. The members of this group are generally destroyed during pasteurization treatments and, therefore, a positive coliform test in the case of pasteurized milk is considered to indicate post-pasteurization contamination.

8.2 The coliform bacteria in milk shall be determined in accordance with the procedure laid down in IS : 5401-1969*.

8.2.1 Since organisms giving positive presumptive tests are practically absent from properly processed pasteurized milk collected directly from pasteurizers, a confirmatory test generally is not needed. Therefore, in routine and official control of pasteurized samples, testing for organisms of the coliform group is limited to the presumptive test.

8.2.2 In presumptive test with liquid media, for routine plant control of pasteurized products where results are likely to be negative, use not less than 3 tubes, each inoculated with 1 ml or, preferably, 10 ml of samples. Where frequent tests for quality control on samples taken at successive one- or two-hour intervals during continuous operation of pasteurizers are almost always negative, use of one tube inoculated with 1 ml, or preferably 10 ml, may be sufficient. In this work, as in obtaining 'estimates' indicating total bacterial contamination, simple examination of routine samples, taken frequently, furnish more useful information than a more complete examination of samples taken less frequently.

8.3 Interpretation

8.3.1 The following coliform contents are suggested as a guide for grading of raw milk supplies:

Raw Milk : 'Coliforms' absent in 1 : 100 dilution — Satisfactory
Pasteurized Milk : 'Coliforms' absent in 1 : 10 dilution — Satisfactory.

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*Methods for detection and estimation of coliform bacteria in foodstuffs.

8.3.2 In case of milk containing added sugar, the results of the presumptive coliform test shall be interpreted with caution because of the possible risk of a false positive.

9. EXAMINATION FOR SPECIAL BACTERIAL GROUPS

9.1 Pathogenic Streptococci

9.1.1 The hæmolytic streptococci of Lancefield's Group A (*Streptococcus pyogenes* Rosenbach) are highly pathogenic for human beings and these may be derived from infected udders of animals or from human carriers coming in contact with milk. Non-pathogenic species of streptococci are common in milk and these should not be confused with pathogenic types. Milk should be examined for beta hæmolytic streptococci in case of (a) samples suspected of having caused septic sore throat, scarlet fever or other human diseases associated with streptococci, and (b) in routine testing of animals in herds producing raw milk.

9.1.2 *Collection of Samples* — For routine herd examinations, collect samples from each quarter of the udder. Where it is not possible to do so, collect group samples from several cows (maximum 10), prepare blood agar plates and examine the plates for beta hæmolytic streptococci. Where a group sample shows presumptive evidence, collect milk from individual cows in the group so that those animals discharging beta hæmolytic streptococci may be eliminated from the herd. Since infections may appear at irregular intervals or may even be overlooked, failure to find infected animals by routine examinations is not complete assurance of the absence of beta hæmolytic streptococci.

9.1.2.1 Where a supply is suspected, collect samples both of well-mixed milk and, if available, gravity-separated cream from each source. When a suspected herd is located, take a sample from each quarter of the udder from the animals in herd. Where examination of samples is delayed, preserve the samples with 30 percent glycerol or 0·000 1 percent brilliant green.

9.1.3 *Apparatus and Materials*

9.1.3.1 *Vials, bottles or test tubes, sterile* — Size 15 to 30 ml or larger containers, glass-stoppered bottles, screw cap bottles or vials, rubber or cork-stoppered test tubes.

9.1.3.2 *Veal (or heart muscle) infusion broth* — Infuse 500 g of finely ground veal (or heart muscle) in one litre of water over night in an ice-box. Boil until the infusion is clear and the coagulum is brown. Strain through cheese-cloth and filter through paper. Add 5 g of sodium chloride and 5 g of tryptone and adjust to *p*H 7·2. Filter the infusion and autoclave at 121°C for 20 minutes.

NOTE — In place of veal infusion, 10 g meat extract may be used.

9.1.3.3 *Veal (or heart muscle) infusion agar* — Prepare veal (or heart muscle) infusion broth containing 2 percent agar. Just prior to pouring plates, add 5 percent of defibrinated sheep, horse or rabbit blood (less is known about the appearance of colonies when bovine blood is used) to the melted medium cooled to 45°C.

NOTE — In place of veal infusion, 10 g meat extract may be used.

9.1.3.4 *Tryptone blood agar* — Prepare medium containing 0·5 percent tryptone, 0·5 percent sodium chloride and 2 percent agar in water. Just prior to pouring plates, add 5 percent of defibrinated rabbit, sheep or horse blood to the melted medium cooled to 45°C. Agar may be stored in small flasks for plating, or in 12- to 15-ml amounts in test tubes. Where Burri agar slants (without added blood) are to be used, slant the tubes and allow them to dry in horizontal position until water of condensation has disappeared.

9.1.4 *Isolation Procedure* — In preliminary routine examinations of milk supplies to detect sources of infection quickly, identification of colonies as beta hæmolytic types is presumptive evidence. Subcultures should be subjected to further identification, supplemented as needed by serological methods.

9.1.4.1 In such preliminary procedures, use blood agar plates or Burri agar slants. In blood agar plate method, just prior to pouring the medium into each plate, add 0·5 ml defibrinated blood to each 10 ml of melted medium cooled to 45°C. Use of Burri agar slants requires less glassware and permits more convenient routine examination of additional samples including samples from each quarter of the udder.

9.1.5 *Blood Agar Plate Method* — When preparing plates, follow the same general procedure as described in **12** of IS : 5409-1969*. To determine sterility of blood, incubate uninoculated blood agar plate controls at 37·0 ± 0·5°C for 24 hours. Also inoculate controls with test organisms which are known to produce beta hæmolysis.

9.1.6 *Burri Agar Slant Method* — With flamed loop, withdraw a loopful of milk and, proceeding from base to top of slant, touch the loop to surface of the medium (without added blood) in three places. Proceeding from base to top, streak inoculum by zigzagging the loop across the surface of slant.

9.1.6.1 Incubate slants at 37·0° ± 0·5°C for 48 hours. Prepare Gram stains of transfers from streptococcus-like colonies. Test the colonies found to be streptococci subsequently in blood agar plates. If such colonies are beta hæmolytic, make complete identification of them. While this procedure is found to be longer than plating directly on blood agar, it selects samples so that only those containing streptococci need be plated. Hæmolytic streptococci have sometimes been found by this method which failed to appear on blood agar plates made directly from original samples. Do not conclude that all hæmolytic colonies are produced by streptococci.

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*Method for plate count of bacteria in foodstuffs.

9.1.7 *Identification of Streptococci, General* — Ordinary milk supplies contain a variety of species of streptococci. *Streptococcus lactis* Lohnis and *Streptococcus cremoris* Orla Jensen, the so-called lactic acid streptococci, have never been isolated from samples drawn aseptically from udders. These species enter milk soon after milking and, if the milk is improperly cooled, grow rapidly. Usually these appear as inactive or green-producing (alpha) types of colonies on blood agar. *Streptococcus agalactiæ*, the most common cause of bovine mastitis, occurs frequently in raw milk and may be abundant in milk from udders in early stages of active infections. On blood agar plates, it produces a green colour and may produce weak hæmolytic (narrow zone) colonies or even broad hæmolyzed zones. This species may be readily differentiated by cultural and serological tests from other related species.

NOTE — *Streptococcus dysgalactiæ* and *Streptococcus uberis* occasionally cause mastitis.

9.1.8 *Beta Hæmolytic Streptococci, Identification*

9.1.8.1 *Presumptive tests* — Examine the plates for typical beta colonies with a clearly defined wide zone of hæmolysis which appear after incubation at 37·0 ± 0·5°C for 18 to 24 hours. Colonies of suspected human origin are small and dense and are surrounded by wide clear zones. Prepare Gram stains of transfers from colonies and from broth cultures to identify organisms as streptococci. Interpret identification of Gram positive, beta hæmolytic streptococcus in freshly drawn quarter samples as presumptive evidence and recommend elimination of infected cows from herd.

9.1.8.2 *Confirmatory tests* — Before condemning a specific milk supply, confirm identity of streptococci by cultural and, where possible, by serological methods.

9.1.9 *Beta Hæmolytic Streptococci in Pasteurized Milk* — Little is known of the origin and significance of beta hæmolytic streptococci frequently found in pasteurized milk which has not been contaminated after processing. At present they are regarded as harmless saprophytes of Lancefield's Group D, among which are *Streptococcus durans* and *streptococcus zymogenes*. They are not regarded as pathogenic for human beings. *Streptococcus* *pyogenes* does not survive pasteurization and accordingly in doubtful cases supplementary tests may be made to determine the heat resistance of cultures.

9.1.10 *Bovine Mastitis Streptococci, Identification* — Many udders discharge streptococci and these streptococci (typically *Streptococcus agalactiæ*, Lancefield's Group B) are usually associated with latent, subclinical or active mastitis and are believed to have little or no direct public health significance. These of bovine origin rarely produce broad zones of hæmolysis around colonies on blood agar plates. Haemolysis is frequently of alpha type. Occasionally on aerobic plates, narrow zones of clear hæmolysis may develop or, by alternating incubation with refrigeration of plates, zones of hæmolysis may appear as double zones. *Streptococcus agalactiæ* hydrolyzes sodium hippurate and produces a final *p*H of 4·2 to 4·6 in one percent glucose broth.

9.2 Tubercle Bacilli

9.2.1 Tubercle bacilli in milk usually are of bovine type. Bacilli of human type may be found in milk but only when grossly contaminated with discharges, especially sputum, from a tuberculous milker or milk handler. Occasionally, some bacilli of avian type may get into milk from faecal or uterine discharges, but this type presents little or no danger to human beings or domestic mammals other than swine.

9.2.1.1 Because tubercle bacilli do not multiply in milk, their presence therein in large numbers indicates gross contamination. Under natural conditions, only bacilli of bovine type derived from tuberculous pus discharged directly into milk from lesions in udders may occur in large numbers. Since tuberculosis localizes in the udder in only a small percentage of cases, large numbers of bacilli are rarely found in market milk. In areas where bovine tuberculosis is common, mixed milk from dairies very commonly contains living tubercle bacilli, which, even though may not be present in large numbers, constitute an important public health menance, especially for children and adolescents.

9.2.1.2 Other possible sources of bovine tubercle bacilli in milk are: (a) excretion by way of blood from tuberculous abscesses of lungs or other organs, (b) contamination from fresh or partly dried faecal material (often rich in bacilli when sputum in advanced pulmonary cases is swallowed) dropping from flanks and udders into milk, and (c) uterine discharges in cases of tuberculosis of the uterus.

9.2.2 *Concentration of Bacilli in Milk* — Because milk usually contains relatively few tubercle bacilli, concentrate organisms present by centrifuging or by placing a sample in refrigerator for at least 24 hours. Fat rises to the surface and most of the foreign matter, such as bits of dirt, manure, leucocytes and fragments of tissue which may have originated in tuberculous ulcers, settles. Most bacteria, including tubercle bacilli, will be found in these two layers. Examine these portions either separately or after mixing.

9.2.2.1 If large quantities of milk are available, allow (0·25 or 0·50 kg) samples to stand for 24 hours in a refrigerator in sterile bottles or cylinders. Collect sediment layers by siphoning. Mix and centrifuge in sterile 250-ml bottles for 30 minutes at high speed. Collect top and bottom layers, mix together, and use the mixture for animal inoculation.

9.2.3 *Methods of Examination* — The three types of methods used for detecting tubercle bacilli in milk are: (a) direct microscopic method, (b) cultural methods, and (c) animal inoculation methods. The average worker should succeed best when using animal inoculation procedure.

9.2.4 *Direct Microscopic Examination* — Tubercle bacilli may be recognized by their acid-fast properties in stained films of milk sediment. When present only in small numbers, they may not be found, and when found, some caution should be observed in identifying them as tubercle bacilli. Many saprophytic acid and alcohol fast organisms exist, some of which, when found in milk, are not readily distinguishable from tubercle bacilli morphologically or tinctorially. Frequently, artifacts may be encountered in cream and mistaken for acid- and alcohol-fast bacilli. Collection of fatty substances around ordinary bacteria or other small objects may so prevent decolourization that the latter resemble acid-fast bacteria. To avoid these effects, rinse the fixed films before staining with a fat solvent, such as ether or xylene.

9.2.4.1 Preferably use Ziehl-Neelsen carbol fuchsine stain prepared in the following manner:

> Dissolve 0·3 g of basic fuchsine (90 percent dye content) in 10·0 ml of 95 percent ethyl alcohol by volume (solution A) and 5·0 of phenol in 95·0 ml of distilled water (solution B). Mix solutions A and B.

9.2.4.2 Stain with steaming carbol fuchsine for at least three minutes. Decolourize for at least 10 seconds in 95 percent alcohol containing 3 percent hydrochloric acid. Counterstain for about 10 seconds in Loeffler's methylene blue solution prepared in the following manner:

> Dissolve 0·3 g of methylene blue (90 percent dye content) in 30 ml of a 5 percent ethyl alcohol and 0·06 g of potassium hydroxide in 100 ml of water and mix the two solutions.

9.2.5 *Cultural Methods* — After destroying nonacid-fast organisms in milk, tubercle bacilli may be cultivated directly from milk at 37°C.

Treatment with hydrochloric acid followed by cultivation on egg medium containing malachite green is used for isolation. Do not regard acid-fast organisms so cultivated as tubercle bacilli without confirmatory evidence.

9.2.6 *Animal Inoculaion Method* — Guinea pigs are highly susceptible to infection with mammalian tubercle bacilli and therefore, are preferred for detecting these bacilli in milk. Since guinea pigs are not highly susceptible to avian bacilli, use rabbits or chickens, if avian type is suspected.

9.2.6.1 Preferably use animals weighing at least 350 g, since small guinea pigs are apt to succumb to the effects of extreneous organisms that may be present in milk. If history of animals to be used is unknown or their infection is suspected, test them intradermally with tuberculin before inoculation.

9.2.6.2 Inoculate animals subcutaneously using up to 5 ml, depending on quality and age of milk. Since dirty or old milk with high bacterial counts is more apt to cause death from peritonitis than better grades, use smaller amounts of the former for injection. Inject at least two animals with each sample. Refrigerate portions of samples so that if all animals inoculated with some sample die within one or two days, other animals may be inoculated with smaller quantities of such samples. If samples are collected especially for examination for tubercle bacilli, and particularly if they are to be shipped, add one percent boric acid to prevent growth of other organisms.

9.2.6.3 Keep inoculated animals in small cages with water-tight bottoms and allow only those inoculated with portions from the same sample to associate.

9.2.6.4 As soon as possible after animals die conduct careful autopsies. Look for caseous masses in the greater omentum and for characteristics lesions in the liver, spleen, and lungs. Tubercle bacilli are not always easily found in stained preparations from these lesions. If the nature of lesions is not clear, inoculate other guinea pigs with them, placing the inoculum in thigh muscles rather than in the peritoneal cavity; infection then may be traced through the lymphatics to visceral organs. Tuberculous lesions may usually be identified by experienced workers even if acid-fast organisms are not demonstrated. In exceptional cases, where doubt exists, cultivate the *Bacillus* on suitable media.

9.2.6.5 After about six weeks, subject test animals to a tuberculin test, by injecting intradermally into the shaved or plucked area of abdomen about 0·1 ml of regular concentrated tuberculin used for testing cattle, and reading such tests after 48 hours. Another method is to inject animals subcutaneously with an equivalent of about 0·5 ml of OT (old tuberculin).

Tuberculous guinea pigs are usually killed by such doses within 12 to 24 hours. Conduct autopsies on animals to detect characteristic lesions. Do not destroy surviving animals until the 8th, or preferably, the 12th week after inoculation.

9.2.6.6 Usually when one animal of a pair becomes tuberculous, the other animal also will. Occasionally, only one animal shows disease, particularly when milk has been underpasteurized; this, no doubt, indicates that very few viable bacilli were present. Infected animals usually show progressive disease with lesions in several internal organs. Occasionally, especially when dealing with milk that has been heated, only a localized infection will be found. Examine such animals carefully, and if evidence of tuberculosis lesion is inconclusive, either inoculate other animals or culture the *Bacillus*. Lesions simulating those of tuberculosis may be produced in experimental animals by injection of any acid-fast organisms, but to produce this effect, saprophytic acid-fast organisms have to be introduced in comparatively large numbers, and probably milk seldom, if ever, contains such numbers. When acid-fast saprophytes are suspended in fatty material, such as cream, as a part of the injected material, their lesion-producing power is enhanced. To detect this type of pseudo-tuberculosis, inject other guinea pigs with material from the lesions. If the lesion is pseudo-tuberculous type, animals will not become infected. Saprophytic acid-fast organisms may also be distinguished by their easy and rapid growth on ordinary culture media on which tubercle bacilli do not grow.

9.3 Brucella Species

9.3.1 *Brucella abortus*, *Brucella suis*, and *Brucella malitensis* are the three species known to be pathogenic for human beings, but so far respective symptom complexes resulting from these infections have not been well differentiated.

9.3.1.1 Human beings may become infected by way of the skin through handling infected animals and their excretions, or by ingesting dairy products containing living organisms. Cows, goats, sheep, horses, dogs, wild deer, wild buffaloes, hogs, and fowls have been naturally infected with *Brucella*. All the three species of *Brucella* have been isolated from infective cow's milk.

9.3.1.2 The milk ring test (*see* **9.3.6**) for determining the presence of brucellosis in cattle may be used advantageously to identify infected herds. Tests are applied to samples of milk from individual herds as delivered at receiving plants.

9.3.2 *Culture and Description of Species* — For primary isolation on culture media, incubate inoculated cultures at 37·0 ± 0·5°C for 5 days both aerobically and in atmosphere containing approximately 10 percent carbon dioxide. If typical colonies develop under aerobic conditions, cultures may be either *Brucella melitensis* or *Brucella suis*. For growing some newly isolated cultures, use of 10 percent carbon dioxide may be required for about 15 successive transfers. When material is cultured from animals inoculated with strains which have become aerobic through successive transfers, use of an increased carbon dioxide atmosphere is unnecessary for primary isolation. Smooth colonies of each of the 3 species appear similar, being spheroidal, 3·5 mm in diameter, slightly bluish and translucent. On direct culture from milk, occasional colonies may be flattened to about 7 mm in diameter. Viewed from above, surface growth on slants appears moist and greasy. Surface growth of *Brucella melitensis* may become brown with age, colour often penetrating below the surface of medium. Colonies should be examined under a low power binocular microscope (about 12 X), using obliquely reflected light. Rough colonies of *Brucella* species are opaque and grey. *Brucella* from smooth colonies possess a capsule, those from rough colonies do not. Optimal growth is at *p*H 6·6 to 6·8.

9.3.3 *Isolation from Milk by Animal Inoculation* — Before examining udders of individual animals, wipe teats with a clean, damp cloth. Discard the first 2 or 3 streams from each quarter. Collect a specimen from the hind quarters in one sterile tube and from the front quarters in another. Take about 8 ml from each quarter. Refrigerate tubes for 24 hours to allow the cream to rise, thereby collecting a majority of organisms in it. Inject the entire cream layer of each sample subcutaneously into a healthy guinea pig. Keep inoculated animals in individual cages. Kill them at the end of six weeks and examine tissues for characteristic lesions produced by *Brucella* species. Make cultures from suspected lesion and test the blood serum of the animals for *Brucella* agglutinins.

9.3.3.1 When examining bottled milk, transfer the entire cream layer to a sterile tube or flask. Inject guinea pigs subcutaneously with 2 ml of thoroughly mixed cream. Where examinations are delayed appreciably by more then one day, preserve each sample before shipment with one percent boric acid or 0·025 percent crystal violet.

9.3.4 *Isolation from Milk by Plate Method* — Prepare medium from ingredients consisting of 2 percent tryptose, 0·1 percent dextrose, 0·5 percent sodium chloride and 1·5 percent agar in water with the final *p*H 6·9 ± 0·1.

9.3.4.1 *Procedure* — The plate method offers a rapid, accurate and quantitative means to detect *Brucella* species in milk drawn directly from udders. Collect milk as in the case of guinea pig inoculation. After refrigerating samples for 24 hours, pipette off cream. Culture by placing 0·1 to 0·2 ml on the surface of each of the two tryptose agar plates containing in final dilution 0·000 14 percent crystal violet. Prepare crystal violet in 0·1 percent aqueous stock solution. By rotating the plate in horizontal plane, spread drops of cream over the surface of the medium with a sterile glass rod bent at right angle. Incubate plates at 37·0 ± 0·5°C for 5 days in an atmosphere containing about 10 percent carbon dioxide. Examine the plates for *Brucella* growths typified as clear light blue-violet colonies, 2 to 7 mm in diameter. Colony counts may range from 0 to more than 1 000 colonies, depending upon the degree of infection. If an inoculated plate contains colonies of other bacteria or moulds, transfer suspicious colonies to tryptose agar slants or to a crystal violet tryptose agar plate.

9.3.4.2 Identify suspicious colonies as *Brucella* species by Gram's staining, biochemical reactions and slide agglutination with known diagnostic *Brucella* agglutination sera. When aerobic types, such as *Brucella suis* or *Brucella melitensis*, are present in milk, incubation in 10 percent carbon dioxide will not inhibit their growth. Aerobic growth of the first transfer of primary culture is presumptive evidence that the species is not *Brucella abortus*. Since strains of *Brucella abortus* which have become aerobic through artificial cultivation may be recovered aerobically from animals following inoculation, other methods have to be used to identify species of newly isolated aerobic stains of *Brucella*. When isolating *Brucella melitensis* from goat or sheep milk, use either of the two methods described for detecting *Brucella abortus* in cow's milk.

9.3.5 *Detection of Brucella Agglutinins in Milk*

9.3.5.1 *Collection and preparation of samples* — Collect samples of milk (avoiding strippings), preferably from each quarter of the udder, in clean test tubes or vials, filling them to about half their volume. Store for 6 to 8 hours and pipette off the cream. Add to each tube two drops of rennet (one percent solution) for each 5 ml of milk, and mix thoroughly. To obtain clear serum incubate tubes in the slanting position for about 2 hours and to reduce particulate casein interference, refrigerate the serum for 6 to 8 hours before testing. Do not use colostrum milk or milk which is sour, decomposed or partially digested.

9.3.5.2 *Agglutination test on milk serum — Tube method* — Obtain standardized *Brucella* agglutinating suspensions from the Indian Veterinary Research Institute, Izatnagar. Place 2 ml of antigen in each of the five agglutination tubes. To the first tube add 0·08 ml of milk serum, to the second 0·04 ml, to the third 0·02 ml, to the fourth 0·01 ml, and to the fifth 0·005 ml, which makes serum dilutions of approximately 1 : 25, 1 : 50, 1 : 100, 1 : 200 and 1 : 400 respectively. Mix and incubate preferably in a water-bath at 37·0 ± 0·5°C for 48 hours. Record the results as follows:

Complete sedimentation	...	Positive
Incomplete sedimentation	...	Doubtful
No sedimentation	...	Negative

NOTE — Complete sedimentation in 1 : 50 or higher dilutions indicates infection in the udder. Run control tests on known positive and known negative samples.

9.3.6 *Milk Ring Test for Brucellosis* — Add standardized antigen consisting of *Brucella* spp cells, stained with haematoxylin, or the tetrazolinium salt [4, 4-bis (3, 5-diphenyl 2-tetrazolinium)-biphenyl dichloride], dropwise in accordance with the directions on the commercial container, to approximately one millilitre of whole milk in a small-bore (about 12 mm) test tube. Mix the contents well and incubate at 37·0 ± 0·5°C for one hour. The mixture has a lighter shade of blue-violet or red than the stained cells, depending partly on the die used. Some laboratories recognize different degrees of colour change and report results in terms of 0, 1 +, 2 +, 3 +, and 4 +.

9.3.6.1 If the sample comes from an infected cow or from pooled milk from a herd containing some infected animals, stained cells rise with the cream layer to form a deeply coloured (either blue-violet or red) ring, leaving the skim milk portion beneath nearly white.

9.3.6.2 If the sample comes from a non-infected cow or from pooled milk from a herd containing no infected animals, the cream layer is either white or only slightly coloured and the colour of skim milk beneath remains practically unchanged.

9.4 Detection and Identification of *Staphylococcus aureus* — Follow the method prescribed in **3** of IS : 5887 (Part II)-1976*.

9.5 Test for Anaerobic Bacteria in Milk (Stormy Fermentation Test) — The presence of anaerobic bacteria in milk (*Clostridum perfringens*, *Clostridum butyricum* and related types) in milk is an indication of manurial or faecal contamination and gives an indirect evidence of insanitary methods of production as well as the possibility of contamination from intestinal pathogens. If aerobic conditions are created by (a) heating milk to remove dissolved oxygen and also to destroy all nonspore-forming bacteria, and (b) by putting a paraffin plug on the surface, these organisms ferment lactose producing acid and gas. As a result, milk will coagulate and the curd particles will be blown apart while the paraffin plug will be pushed up.

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*Methods for detection of bacteria responsible for food poisoning: Part II Isolation, identification and enumeration of *Staphylococcus aureus* and Faecal steptococci (*first revision*).

10. DETECTION OF MASTITIS

10.1 Mastitis or inflammation of the udder is caused by certain species of streptococci (principally *Streptococcus agalactiae*), staphylococci and occasionally by *Corynebacterium pseudomonas*, coliforms and actinomycetes. Though in most cases mastitis is caused by streptococci, non-pathogenic for human beings, their occurrence interferes with the efficient secretion of milk and is invariably a source of great economic loss. It is, therefore, necessary to regularly examine all the milking animals in a herd to ensure that none are suffering from mastitis, and whenever positive cases are detected, to take prompt measures to check and eliminate the disease.

10.2 It is essential that the tests for the detection of mastitis should be made on single quarter samples since milk from normal quarters may obscure changes of the abnormal ones if composite samples are taken. Any quarter giving an abnormal reaction should then be followed up. The first 2 or 3 streams of milk should always be used for diagnostic tests as these will show the greatest change if the quarter is abnormal.

The rapid tests given in **10.2.1** to **10.2.6** are useful for detecting mastitis at different stages. Usually, it is advisable to employ a combination of several of these tests and to regard the results as preliminary or presumptive, to be confirmed by more exact tests, such as isolation of the causative organisms.

10.2.1 *The Strip-Cup Test* — A piece of finely woven black cloth, or screen, stretched lightly over a cup and held in place by a rubber band will serve as a satisfactory strip-cup. Strip-cups provided with four compartments so that milk from each quarter is collected in a separate compartment are obviously advantageous to use. Milk the first two or three streams of milk on to the cloth (or screen) and observe for any clots or mucus. Positive findings are quite conclusive evidence of mastitis. Milk from such animals should be excluded. A negative reaction does not mean absence of infection, since only acute cases will be detected on only one examination.

10.2.2 *Bromothymol Blue Test* — This test is based on the fact that milk from infected udders is usually alkaline and shows *p*H as high as 7·4. A ready-made indicator solution supplied for *p*H test may be used along with the technique prescribed by the manufacturer. Alternatively, the indicator solution is prepared by titrating 1 g of bromothymol blue powder with 160 ml of 0·01 N sodium hydroxide solution and diluting with 590 ml of distilled water. Place 1 ml of bromothymol blue solution in a test tube and add 5 ml of milk. Mix and observe the colour.

10.2.2.1 *Interpretation* — Bromothymol blue added to normal milk gives a yellow colour. Milk from infected quarters will show a green to a slightly bluish-green colour depending upon the degree of alkalinity. Although this test will detect most acute or subacute cases, many chronic cases will not show sufficient change in *p*H to be detected by the method. Cows which are nearing the lactation period may give positive tests and not be infected.

10.2.3 *Bromocresol Purple Test* — This indicator has the advantage over bromothymol blue in that it has a yellow range below *p*H 5·2. Consequently, milk which is abnormally acidic may also be detected. Such milk may occasionally come from infected udders. A ready-made indicator solution may be used according to the instruction of the manufacturer. Alternatively, 0·5 g of bromocresol purple is dissolved in 100 ml of water. To 9·5 ml of milk add 0·5 ml of the indicator solution. Mix and observe the colour. Normal milk is coloured a pale greyish-purple. Abnormal milk becomes a deep purple with increased alkalinity.

NOTE — Blotting papers soaked with indicator solutions and dried may also be used under field conditions. A drop of milk is placed on the paper and the colour observed.

10.2.4 *Catalase Test*

10.2.4.1 Most living cells including leucocytes contain the enzyme catalase. Udder infection increases the number of leucocytes in the milk, and the catalase test is used to measure the increase depending on the ability of the enzyme to break down hydrogen peroxide.

10.2.4.2 *Procedure* — Place 15 ml of freshly drawn milk in a 20-ml graduated test tube, and fill the tube completely with 1 percent hydrogen peroxide solution. Close the tube with a stopper having a hole through which is fitted a piece of glass tubing bent in S-shape. The tube is inverted, placed in a suitable beaker, and incubated at 37·0 ± 0·5°C for 3 hours. The oxygen liberated by the action of catalase collects in the upper portion of the test tube.

10.2.4.3 *Interpretation* — In normal milk the amount of oxygen thus liberated will be about 2·0 ml. More than 2·5 ml of gas collected in the top of the tube is presumed to be due to abnormal infected udder. Milk from chronic cases may produce as much as 10 ml of gas. Milk of animals in early or late lactation may give positive tests to catalase and be free of infection.

10.2.5 *Chloride Test*

10.2.5.1 The chloride content increases in milk of animals suffering from mastitis.

10.2.5.2 *Procedure* — To accurately measured 5 ml of 0·134 1 percent silver nitrate solution, add two drops of 10 percent potassium chromate indicator. This gives a red colour. To the mixture add exactly 1 ml of milk and mix. If the sample contains an abnormally high percentage of chloride, the red colour will change to yellow. A brownish-red colour indicates a negative test. Animals in early, or in late lactation may give false positive reaction to chloride test.

10.2.6 *Hotis Test*

10.2.6.1 Amongst the tests described, Hotis test gives the most accurate information about mastitis infection. It is based on the fact that *Streptococcus agalactiae* when growing in milk, produces a characteristic colony like mass of cells adhering to the sides of the test tube. By the introduction of an acid indicator (bromocresol purple), the identification of these colonies, or ' buttons ' is facilitated by their characteristic yellow colour.

10.2.6.2 *Procedure* — Place 0·5 ml of bromocresol purple solution (0·5 g in 100 ml of water) in a test-tube. Add 9·5 ml of milk drawn aseptically. Mix and incubate at 37·0 ± 0·5°C for 24 hours. The appearance of canary yellow colonies of organisms along the sides of the tube or on the bottom indicates infection with *Streptococcus agalactiae*. Since acid is produced by the organisms in these colonies, the reaction of milk immediately surrounding the colony changes first. The yellow colour of the colonies along the sides of the tube contrasts with the purple background during the first 24 hours of incubation.

NOTE — If more than one kind of organisms is present in the infected quarter, or if the tests are contaminated during their collection, a combination of changes may obscure the typical Hotis reaction.

APPENDIX A

(*Clause* 0.4)

INDIAN STANDARDS ON INGREDIENTS USED IN MEDIA FOR MICROBIOLOGICAL WORK

IS : 6850-1973	Agar, microbiological grade
IS : 6851-1973	Meat extract, microbiological grade
IS : 6852-1973	Bile salts, microbiological grade
IS : 6853-1973	Peptone, microbiological grade
IS : 6854-1973	Methods of sampling and test for ingredients used in media for microbiological work
IS : 7004-1973	Yeast extract, microbiological grade
IS : 7127-1973	Tryptone microbiological grade
IS : 7128-1973	Proteose peptone, microbiological grade
IS : 7203-1973	Casein hydrolysate (acid digested), microbiological grade
IS : 7535-1975	Liver extract, microbiological grade
IS : 7536-1975	Soluble starch, microbiological grade
IS : 7590-1975	Gelatin, microbiological grade
IS : 7591-1975	Malt extract, microbiological grade
IS : 7801-1975	Trypsin, microbiological grade

(*Continued from page* 2)

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